INHIBITION BY PENICILLIN OF THE INCORPORATION AND CROSS-LINKING OF L-LYSINE IN INTACT CELLS OF *MICROCOCCUS LUTEUS*

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1. Introduction

A generally accepted theory is that penicillin kills bacteria by inhibiting the transpeptidation reaction which leads to cross-linking of peptide side chains in the growing bacterial cell wall glycopeptide. This conclusion has been based on studies both in vitro and in vivo in a limited number of organisms, mainly *Escherichia coli* [1, 2], *Bacillus megaterium* [3], and *Staphylococcus aureus* [4–6].

Using a novel in vitro crude enzyme which consists of cell walls of *Micrococcus luteus* (*lysodeikticus*) containing a strongly associated membrane fraction, we have recently shown that penicillin inhibits not only the transpeptidation reaction but also the covalent binding of newly synthesised peptidoglycan to the pre-existing cell wall [7, 8]. On the basis of these and related findings, we have concluded that the transpeptidase functions both for cross-linking and for the incorporation proper of linear peptidoglycan strands into the cell wall. It thus appears that elongation of the pre-existing polymer is the result of two enzyme-catalysed incorporation reactions, the penicillin-insensitive transglycosylation and the penicillin-sensitive transpeptidation [7, 8].

In this communication, we present new data obtained with intact cells of *M. luteus* which support these conclusions. We show that these cells incorporate radioactive L-lysine into their cell wall peptidoglycan and that the incorporation is inhibited by penicillin G. Approximately 40% of the radioactive lysine incorporated into the cell wall in the absence of penicillin, participates in the formation of peptide cross-links via its ϵ-amino moiety, whereas in the presence of penicillin no formation of such cross-links occurs.

2. Methods

The in vivo incorporation of radioactive L-lysine into cells of *M. luteus* NCTC 2665 was studied in a minimal medium in which cell wall synthesis can take place [9]. For this purpose early logarithmic phase cells were used. The cells were grown at 32°C in 0.5% bactopeptone (Difco), 0.5% NaCl, 0.2% yeast extract and 1% glucose, harvested at 0.5 absorbance (Zeiss spectrophotometer, 660 nm), and washed once with phosphate buffer (0.05 M, pH 7.0). The cells thus prepared (2.5×10^{10}) were transferred to 50 ml of a minimal medium containing glycine (1 mM), L-glutamic acid (1 mM), L-alanine (0.5 mM), glucose (0.0285 M), together with salts and cofactors as described [9]. Chloramphenicol (70 μg/ml) was added to the medium, and after incubation for 5 min with shaking at 32°C, uniformly labeled L-[^{14}C]lysine (Radiochemical Centre, Amersham, >99.9% pure, specific activity, 342 mCi/mM, diluted with unlabeled L-lysine to a specific activity of 7.5×10^2 cpm/nmoles) was added to the medium to a final concentration of 20 nmoles/ml. The radioactivity was routinely determined by counting in an ethoxyethanol-containing fluid [10], with a counting efficiency of 71%. Incubation with shaking was continued for predetermined periods of time (up to 30 min). The cells were then harvested, thoroughly washed with phosphate buffer (5 × 50 ml, 0.05 M, pH 7.2), and disintegrated in water (25 ml) with glass beads (25 g) in a Braun homogeniser.

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The cell walls (about 2 mg/50 ml culture) were isolated by differential centrifugation and extensively purified by heating with 1% sodium dodecyl sulfate in water and then washing in water as described [11]. Amino acid analysis of the purified walls showed that more than 97% of the amino acids detected, belong to the cell wall peptidoglycan.

To estimate the extent of cross-linking of the L-[^{14}C]lysine incorporated into the walls, analysis of [^{14}C]lysine in acid hydrolysates was carried out on cell walls obtained from cells grown in the absence or presence of penicillin G (10 μg/ml) both before and after deamination of the intact walls [12]. For deamination, a portion (1 mg) of the purified walls was treated with $NaNO_2$ (1.0 M) in acetic acid (1.0 M) for 2 hr at 23°C in a total volume of 0.5 ml. The deaminated walls were washed with water by centrifugation (4 X 1 ml; 12 000 *g*) and then hydrolysed (6 N HCl, 20 hr, 100°C).

Analysis of the radioactive compounds in the cell wall hydrolysates was carried out on the short column (6 cm) of the amino acid analyser (Beckman 120°C), using citrate buffer (0.2 M, pH 5.25) as eluent. The effluent of the amino acid analyser column was directly connected to a flow scintillation spectrometer (Packard Tri-Carb 3022) calibrated with pre-determined amounts of L-[^{14}C]lysine. A standard of radioactive α-amino-ϵ-hydroxy caproic acid was prepared by nitrous acid deamination under the above conditions of UDP–MurNAc–L-Ala–D-Glu–L-[^{14}C]Lys–D-Ala–D-Ala which was prepared according to the literature [9]. On the amino acid analyser, α-amino-ϵ-hydroxy caproic acid is eluted from the column after 5 min whereas unmodified lysine is eluted after 17 min.

Digestion of cell walls with lysozyme was performed as before [13]. Digests were separated by paper electrophoresis (50 V/cm, 90 min) in pyridine (0.2 M), adjusted with acetic acid to pH 6.5 [13], and by chromatography (72 hr) in *n*-butanol:acetic acid:water (4:1:5, upper phase).

3. Results

The incorporation of L-[^{14}C]lysine into the cell walls was time dependent and markedly inhibited by low concentrations of penicillin G (about 70% inhibition at 10 μg/ml) (fig. 1). In the absence of penicillin, 100 nmoles of lysine were incorporated within 30 min into the cell walls of bacteria obtained from 50 ml of the defined medium. Since lysine comprises about 10% of the weight of the cell wall [13] there was, in these experiments, approximately a 5% increase in the weight of the cell wall peptidoglycan, whereas in the presence of penicillin, the increase within 30 min was only about 1.5%.

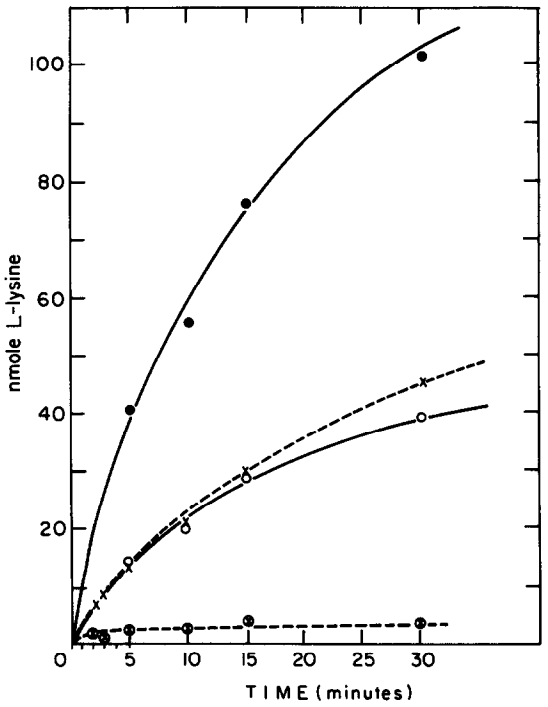


Fig. 1. Rate of L-[^{14}C]lysine incorporation into cell wall of *M. luteus* in the absence and presence of penicillin, and the rate of cross-linking of the ϵ-amino groups of L-[^{14}C]lysine in both cases. Results are expressed as the amount of [^{14}C]lysine found in walls (~ 2 mg) isolated from 50 ml cultures before and after deamination (for details, see text). 'Time' refers to incubation in the presence of radioactive lysine. (●——●——●), L-[^{14}C]lysine incorporated into the cell wall in the absence of penicillin. (○——○——○), L-[^{14}C]lysine incorporated into the cell wall in the presence of penicillin G (10 μg/ml); (X---X---X), [^{14}C]lysine recovered after deamination and hydrolysis of cell walls; (⊕---⊕---⊕), [^{14}C]lysine recovered after deamination and hydrolysis of cell walls, obtained from cells which incorporated L-[^{14}C]lysine in the presence of penicillin G.

In acid hydrolysates of deaminated walls isolated from organisms grown in the absence of penicillin, about 55–60% of the radioactivity was found in a compound which migrated as α-amino-ϵ-hydroxy caproic acid on the short column of the amino acid analyser (fig. 2 A,B). The remaining 40–45% of the

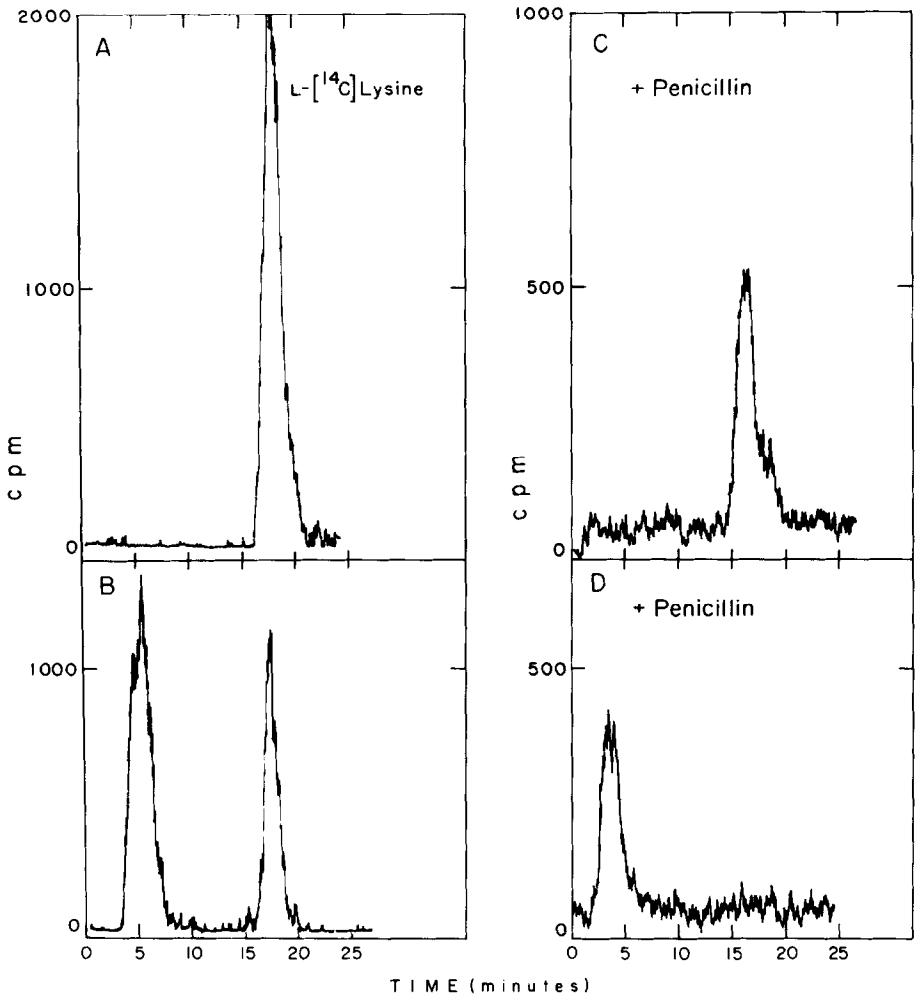


Fig. 2. Flow scintillation charts of the effluent from the amino acid analyser short column (for conditions see text): (A) Acid hydrolysate of cell walls labeled by L-[^{14}C]lysine in vivo for 30 min; (B) hydrolysate of the same cell walls deaminated with HNO_2 prior to hydrolysis. Labeled material emerging from the column after 5 min, has its ϵ-amino group deaminated; material emerging after 17 minutes is unmodified L-[^{14}C]lysine; (C) hydrolysate of cell walls labeled with L-[^{14}]lysine in vivo in the presence of penicillin G (10 μg/ml); (D) hydrolysate of same walls as in C deaminated prior to hydrolysis. Note the difference in scale of ordinate in (A) and (B) as compared to (C) and (D).

radioactivity was recovered as unmodified [^{14}C]lysine. When the L-[^{14}C]lysine was incorporated in the presence of penicillin G, more than 95% of the ϵ-amino groups of L-[^{14}C]lysine were deaminated (fig. 2, C,D) (table 1), indicating the absence of cross-linking under these conditions.

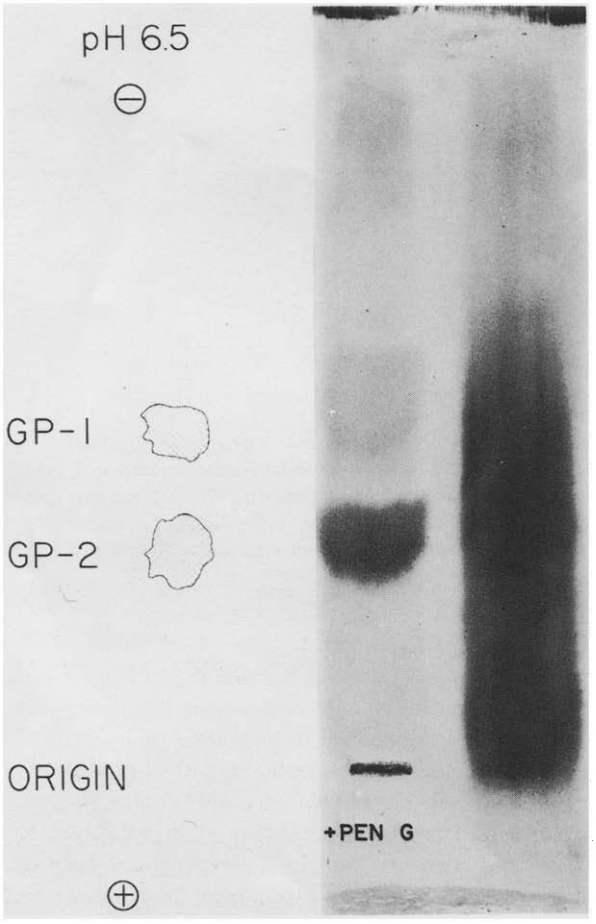
The rate of cross-linking of the L-[^{14}C]lysine paralleled the rate of incorporation of L-[^{14}C]lysine into the cell wall (fig. 1). However, almost no cross-linking of the newly incorporated lysine was detected when incorporation was carried out in the presence of penicillin (figs. 1 and 2).

Lysozyme digests of cell walls labeled in vivo with L-[^{14}C]lysine afforded a variety of radioactive glycopeptides when subjected to separation by paper electrophoresis (fig. 3). On the other hand, walls similarly labeled in the presence of penicillin gave, upon lysozyme digestion, only one type of radioactively-labeled glycopeptide fragment, which migrated on electrophoresis as the uncross-linked disaccharide-pentapeptide GlcNAc–MurNAc–Ala–Glu(Gly)–Lys–

Table 1
Effect of penicillin G on cross-linking at the ϵ-NH_2 moiety of L-[^{14}C]lysine in intact cells.

Experiment	Penicillin G (10 μg/ml)[a]	Deamination of isolated cell walls[b]	Radioactive compound in cell wall hydrolysate[c]	
			[^{14}C]lysine	[^{14}C]α-amino, ϵ-hydroxy caproic acid
			cpm/250 μg walls	cpm/250 μg walls
1	–	–	9500	–
2	–	+	4080 (43%)	5160 (54%)
3	+	–	3410	–
4	+	+	> 200	3100 (91%)

[a] Incorporation of L-[^{14}C]lysine was done in a minimal medium for 30 min. For conditions see text.
[b] Deamination was carried out with $NaNO_2$ as described in text.
[c] Separation of radioactive compounds was on the short column of the amino acid analyser (6 cm) using citrate buffer (0.2 M, pH 5.25) as eluent. For analysis, a sample equivalent to 250 μg cell walls was used. For additional details, see text.



Ala (GP-2, ref. [13]) (fig. 3). Paper chromatograms of lysozyme digests of the radioactive cell walls clearly showed that cross-linking occurred as early as 2 min after the addition of L-lysine to the medium. Moreover, penicillin inhibited the cross-linking of the incorporated lysine even at this early stage and no cross-linked fragmanet GP-1 [13] could be detected on the chromatograms (fig. 4).

4. Discussion

It has been reported that penicillin inhibited considerably the incorporation of radioactive amino acids into the cell wall peptidoglycan of intact cells [4, 6, 14–16]. Furthermore, it has been shown that in cells of *S. aureus*, grown in the presence of penicillin, no marked formation of uncross-linked peptidoglycan took place in the cell walls [4–6]. These phenomena have not been completely understood, mainly because no in vitro inhibition by penicillin of linear peptidoglycan synthesis was ever observed [9, 17–20].

Fig. 3. Paper electrophoresis autoradiogram of lysozyme digests of cell walls labeled with L-[^{14}C]lysine in the presence or absence of penicillin G (10 μg/ml). For conditions see text. GP-2 is the disaccharide–pentapeptide GlcNAc–MurNAc–Ala–Glu(Gly)Lys–Ala and GP-1 is its cross-linked dimer [13].

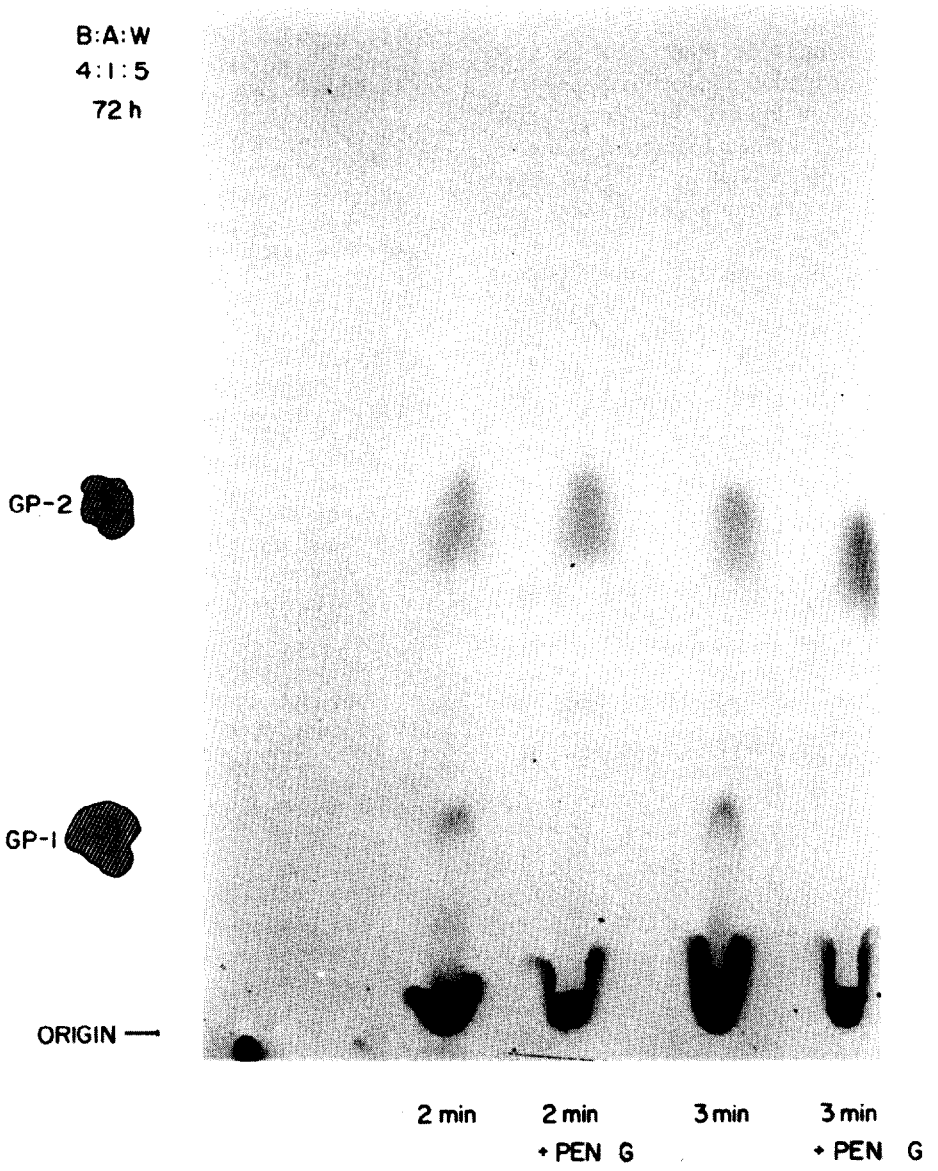


Fig. 4. Paper chromatography autoradiogram of lysozyme digests of cell walls labeled with L-[^{14}C]lysine for 2 or 3 min in the presence or absence of penicillin G (10 μg/ml). For conditions see text. GP-2 is the disaccharide pentapeptide and GP-1 is its cross-linked dimer (see fig. 3). Under these chromatographic conditions, the high molecular material remaining after lysozyme digestion does not migrate [13].

These findings can now be explained on the basis of our proposal that the penicillin-sensitive transpeptidase functions in the insertion and covalent binding of newly synthesized linear peptidoglycan strands into the preformed cell wall. Thus, inhibition of transpeptidation in vivo will result, among other findings in the reduction of the incorporation of cell wall constituents such as lysine or other amino acids, into the cell wall peptidoglycan. Penicillin apparently does not inhibit the transglycosylation reaction, and incorpora-

tion of cell wall precursors without cross-linking can therefore proceed, though to a limited extent only (about 30%), even at high concentrations of the antibiotic (10 μg/ml, which are equivalent to 200 times the MIC in the case of *M. luteus*). This incorporation appears to be the result of linear elongation of the glycan strands by transglycosylation of oligosaccharide–peptide intermediates to the non-reducing ends of pre-existing polysaccharide chains.

It seems likely, therefore, that cell wall elongation and growth is the result of a concerted operation by both (a) transpeptidation as a mechanism for attaching newly synthesized strands to free amino groups of the existing peptidoglycan and (b) transglycosylation for the elongation of the glycan chains.

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